

12/ppts

METHODS AND COMPOSITIONS FOR THE  
TREATMENT AND PREVENTION OF GRAFT REJECTION  
USING HEAT SHOCK PROTEINS

This application is a continuation-in-part of co-pending  
5 application number 09/393,652, filed September 10, 1999,  
which is hereby incorporated by reference in its entirety.

This invention was made with government support under  
grant numbers CA44786 and CA64394 from the National  
Institutes of Health. The government has certain rights in  
10 the invention.

1. INTRODUCTION

The present invention relates to methods for treatment  
and prevention of graft rejection, e.g., in response to  
tissue or organ transplantation. In the practice of the  
15 treatment and prevention of graft rejection, compositions of  
complexes of heat shock/stress protein (hsps) including, but  
not limited to, hsp70, hsp90, and gp96, either alone or in  
combination with each other, noncovalently bound to antigenic  
molecules, are used to suppress the immune response to the  
20 grafted tissue or organ. In addition, compositions  
containing un-complexed stress proteins (i.e., free of  
antigenic molecules) are also used to suppress the immune  
response to the grafted tissue or organ. The invention  
encompasses administration of heat shock proteins before,  
25 after, or both before and after transplantation or grafting.  
In addition, the invention encompasses administration of  
donor tissue sample prior to administration of heat shock  
protein and subsequent transplantation or grafting.

2. BACKGROUND OF THE INVENTION

30 2.1. The Immunology of Transplant and Graft Rejection

Organs are transplanted clinically to rectify an  
irreversible functional deficit but, unless donor and  
recipient are genetically identical, graft antigens will  
trigger a rejection response by the recipient. The study of

skin graft rejection in mice led to the discovery of the major histocompatibility complex (MHC) antigens, the function of which is to bind processed antigens and present them to T lymphocytes. T lymphocytes are pivotal in transplant rejection. The sensitization phase of rejection is due mainly to passenger leucocytes in the graft being recognized as foreign by the recipient's CD4+ T cells. The effector phase of rejection involves these activated recipient T cells entering the graft and locally producing cytokines. The rate of rejection depends on the relative contribution of the underlying immunological effector mechanisms. The pursuit of prevention of rejection has led to the development and use of new immunomodulating agents, approaches which have implications in the treatment of many other immunological disorders. For a review of the immunological background to transplantation, see Haeney, M., 1995, J. Antimicrob. Chemother. 36 Suppl. B:1-9.

Organ transplantation is now the treatment of choice for end stage organ failure. The ultimate goal in transplantation has been the development of strategies to induce specific tolerance to the allograft. The MHC antigens are the principal targets of the immune response to allografts and T cell recognition of allo-MHC is the initial event which initiates allograft rejection. The availability of sequences of MHC genes in mice, rats, and humans has made it possible to prepare synthetic peptides for the study of the role of MHC peptides in allorecognition and tolerance induction. There are at least two distinct, but not necessarily mutually exclusive, pathways of allorecognition. In the so-called "direct" pathway, T cells recognize intact allo-MHC molecules on the surface of donor cells. These MHC molecules contain an array of endogenous peptides bound in their antigen presentation groove. In the "indirect" pathway, T cells recognize specific processed alloantigen presented as peptides in the context of self MHC by antigen-presenting cells (APCs). In addition, there is evidence that synthetic MHC peptides can immunomodulate the alloimmune response both in vitro and vivo, and that

allo-tolerance can be induced with synthetic MHC peptides. Two types of effects mediated by synthetic MHC peptides have been reported: (1) suppression of the alloimmune response by relatively non-polymorphic peptides and (2) antigen-specific unresponsiveness induced by polymorphic peptides. For a review of these mechanisms mediating the immunomodulatory effects of synthetic class I and class II MHC peptides and the potential for clinical applications, see Sayegh, M et al., 1996, Kidney Int. Suppl. 53:S13-20.

10           2.2. Hsps in Graft Rejection

Hsps in grafted tissue have been suggested to be alloantigenic targets of heart graft rejecting immune responses. Qian et al., 1995, Transplant Immunology 3: 114-123, reported elevated hsp expression in cardiac allografts in mice. Qian et al. also reported the presence of infiltrating lymphocytes reactive with mycobacterial hsp60 and hsp70 and with murine grp78 in cardiac allografts undergoing rejection. Moliterno et al., 1995, J. Heart Lung Transplant. 14: 329-337 also reported that anti-hsp60 autoimmune T cells accumulate at sites of inflammation in transplanted heart.

Chaperonin 10 (cpn10) has also been referred to as early pregnancy factor (EPF). Cpn10 is homologous to the heat shock protein groES. Administration of cpn10 following skin grafts was reported to significantly prolong the viability of allogenic skin grafts in rats (International Publication Nos. WO 95/15338 and WO 95/15339).

Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for the treatment and prevention of graft rejection. Treatment regimens include the administration of heat shock proteins (hsps). Because the protection is based on the immunoregulatory role of the hsp itself (and not its

antigenicity), the effectiveness of the treatment is general -- unlike free peptide or other specific graft alloantigen approaches (including where the hsp itself is an alloantigen), the treatment is not limited to a specific target alloantigen of the rejection process. The effectiveness of the hsp administration is not dependent on identity between the organ or tissue from which the hsp was obtained and the tissue or organ which is being transplanted. The hsp-mediated suppression of graft rejection may be dependent on the pre-existing development of the graft-specific autoimmune attack. Thus, the source of hsp does not require tissue specificity in order to effect suppression because its suppressive activity may attain specificity by acting against a previously activated T cell response, which is specific. Accordingly, the treatment regimens disclosed are useful for the treatment and prevention rejection of a variety of grafted tissues and organs. The example in Section 6, below, demonstrates in detail the effectiveness of prevention of skin graft rejection using the heat shock protein gp96.

The treatment methods of the invention are more specific than common cytokine approaches to induction of suppression which are excessively systemic. The hsps used in accordance with the invention exert a more local and targeted immunosuppressive effect at the site of immune cellular activity.

Hsps may be administered, in accordance with the invention, before, after, or both before and after transplantation or grafting. In addition, the invention encompasses administration of donor tissue sample prior to administration of heat shock protein and subsequent transplantation or grafting.

Particular compositions of the invention and their properties are described in the sections and subsections which follow. The invention provides methods for determining doses of hsp administered for treatment and prevention of graft rejection. In general, the dosages required for suppressing the immune response are higher than those typically used for generating an immune response. In

addition, the invention provides pharmaceutical formulations for administration of the compositions in appropriate dosages. The invention also provides routes of administration of the compositions used for treatment and prevention of graft rejection.

In another embodiment the invention encompasses a method of preventing or treating rejection of a grafted cell, tissue, or organ in a mammal comprising administering to the mammal a composition comprising a purified complex consisting essentially of a heat shock protein noncovalently bound to an antigenic molecule, wherein the composition does not comprise a heat shock protein that is an alloantigen of the grafted cells, tissue, or organ.

In another embodiment, the invention encompasses a method of preventing or treating rejection of a grafted cell, tissue, or organ in a mammal comprising administering to the mammal a composition comprising a purified complex consisting essentially of a heat shock protein noncovalently bound to an antigenic molecule, wherein the composition does not comprise any of said complex wherein said antigenic molecule is an alloantigen of the grafted cells, tissue, or organ.

The example presented in Section 6, below, demonstrates the use of compositions comprising gp96 in the prevention of skin graft rejection.

#### 4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Summary of results of skin graft Experiment 1. Results from Day 14 (4 days after engraftment); Day 15, (5 days after engraftment; Day 17 (7 days after engraftment); Day 18 (8 days after engraftment); Day 19 (9 days after engraftment); and Day 20 (10 days after engraftment) are each shown as five rows of five ovals. Each oval represents the skin graft of a different mouse. For each day's results, the rows correspond to five different mice each treated as follows:

- 1<sup>st</sup> Row: Buffer (phosphate buffered saline), alone;
- 2<sup>nd</sup> Row: 100  $\mu$ g gp96 isolated from donor liver;
- 3<sup>rd</sup> Row: 100  $\mu$ g gp96 isolated from donor skin;

- 4<sup>th</sup> Row: 100  $\mu$ g gp96 isolated from donor liver and 100  $\mu$ g gp96 isolated from donor skin; ---
- 5<sup>th</sup> Row: a mixture of donor liver and skin cell lysates.

5 The condition of the skin graft is depicted in each oval using markings as follows:

- Blank = Graft tissue healthy;  
 Speckles = Graft tissue red/inflamed;  
 Black = Graft tissue dead;  
 10 X = Animal died.

FIG. 2. Summary of results of skin graft Experiment 2. Results from Day 18 (8 days after engraftment); Day 22, (12 days after engraftment); Day 24 (14 days after engraftment); Day 18 (8 days after engraftment); Day 26 (16 days after engraftment); and Day 29 (19 days after engraftment) are each shown as five row of two ovals. Each oval represents the skin graft of a different mouse. For each day's results, the rows correspond to two different mice each treated as follows:

- 20 1<sup>st</sup> Row: No treatment;  
 2<sup>nd</sup> Row: Buffer administered intradermally;  
 3<sup>rd</sup> Row: Buffer administered subcutaneously;  
 4<sup>th</sup> Row: 1  $\mu$ g gp96 administered intradermally;  
 5<sup>th</sup> Row: 10  $\mu$ g gp96 administered intradermally;  
 25 6<sup>th</sup> Row: 10  $\mu$ g gp96 administered subcutaneously;  
 7<sup>th</sup> Row: 100  $\mu$ g gp96 administered intradermally;  
 8<sup>th</sup> Row: 100  $\mu$ g gp96 administered subcutaneously;  
 9<sup>th</sup> Row: 200  $\mu$ g gp96 administered subcutaneously;  
 10<sup>th</sup> Row: 10  $\mu$ g rat gp96 administered intradermally.

30 The condition of the skin graft is depicted in each oval using markings as follows:

- Black = Graft tissue necrotic;  
 Hatched = Graft tissue hemorrhagic;  
 Blank = Graft fallen off, underlying wound visible;  
 35 Lightly Speckled = Graft tissue healthy;  
 Heavily Speckled = Graft tissue less healthy area;  
 Single Diagonal Slash = Animal died.

FIGS. 3A-B.. Results of Day 18 (8 days after engraftment) from skin graft Experiment 2, presented as described for FIG. 2, above.

5 FIGS. 4A-B. Results of Day 22 (12 days after engraftment) from skin graft Experiment 2, presented as described for FIG. 2, above.

FIGS. 5A-B. Results of Day 24 (14 days after engraftment) from skin graft Experiment 2, presented as described for FIG. 2, above.

10 FIGS. 6A-B. Results of Day 26 (16 days after engraftment) from skin graft Experiment 2, presented as described for FIG. 2, above.

15 FIGS. 7A-B. Results of Day 29 (19 days after engraftment) from skin graft Experiment 2, presented as described for FIG. 2, above.

#### 5. DETAILED DESCRIPTION OF THE INVENTION

Methods and compositions for the treatment and prevention of graft rejection are described. The invention is based, in part, on newly discovered immunotherapeutic and immunoprophylactic treatment regimens for graft rejection. In contrast to other methods reported in the literature, the use of hsps in accordance with the present invention are not dependent on administration of any particular target antigen of the rejection process.

25 "Graft" and "Transplant" are used interchangeably herein and each encompass the transfer of cells, tissues, or organs from one location to another, including from one individual to another individual.

30 "Antigenic molecule" as used herein refers to any molecule noncovalently bound to a heat shock protein, including, but not limited to, the peptides with which the hsps are endogenously associated *in vivo* as well as exogenous antigens/immunogens (*i.e.*, with which the hsps are not

complexed *in vivo*) or antigenic/immunogenic fragments and derivatives thereof.

5 The hsps of the present invention that can be used include but are not limited to, gp96, hsp90, and hsp70, either alone or in combination with each other. Preferably, the hsps are mammalian hsps. More preferably, for the treatment or prevention of graft rejection in humans, the hsps are human hsps.

10 In a particular embodiment, the hsp is not cpn10. In yet another particular embodiment, the hsp is not hsp60.

Heat shock proteins, which are also referred to interchangeably herein as stress proteins, useful in the practice of the instant invention can be selected from among any cellular protein that satisfies any one of the following  
15 criteria. A heat shock protein is characterized by having its intracellular concentration increase when a cell is exposed to a stressful stimulus, by being capable of binding other proteins or peptides, and by being capable of releasing the bound proteins or peptides in the presence of adenosine  
20 triphosphate (ATP) or low pH, or by having at least 35% homology with any cellular protein having any of the above properties.

The first stress proteins to be identified were the heat shock proteins (hsps). As their name implies, hsps are  
25 synthesized by a cell in response to heat shock. To date, three major families of hsp have been identified based on molecular weight. The families have been called hsp60, hsp70 and hsp90 where the numbers reflect the approximate molecular weight of the stress proteins in kilodaltons. Mammalian  
30 hsp90 and gp96 each are members of the hsp90 family. Many members of these families were found subsequently to be induced in response to other stressful stimuli including, but not limited to, nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens.  
35 (See Welch, May 1993, *Scientific American* 56-64; Young, 1990, *Annu. Rev. Immunol.* 8:401-420; Craig, 1993, *Science* 260:1902-1903; Gething, et al., 1992, *Nature* 355:33-45; and Lindquist, et al., 1988, *Annu. Rev. Genetics* 22:631-677), the



disclosures of which are incorporated herein by reference. It is contemplated that hsps/stress proteins belonging to all of these three families can be used in the practice of the instant invention.

5       The major hsps can accumulate to very high levels in stressed cells, but they occur at low to moderate levels in cells that have not been stressed. For example, the highly inducible mammalian hsp70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized  
10 proteins in the cell upon heat shock (Welch, et al., 1985, *J. Cell. Biol.* 101:1198-1211). In contrast, hsp90 and hsp60 proteins are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat (Lai, et al., 1984, *Mol. Cell. Biol.* 4:2802-10; van Bergen en  
15 Henegouwen, et al., 1987, *Genes Dev.* 1:525-31).

Heat shock proteins are among the most highly conserved proteins in existence. For example, DnaK, the hsp70 from *E. coli* has about 50% amino acid sequence identity with hsp70 proteins from excoariates (Bardwell, et al., 1984, *Proc. Natl.*  
20 *Acad. Sci.* 81:848-852). The hsp60 and hsp90 families also show similarly high levels of intra families conservation (Hickey, et al., 1989, *Mol. Cell. Biol.* 9:2615-2626; Jindal, 1989, *Mol. Cell. Biol.* 9:2279-2283). In addition, it has  
25 been discovered that the hsp60, hsp70 and hsp90 families are composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stress. Therefore it is contemplated that the definition of stress protein, as used herein, embraces other proteins,  
30 muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with members of the three families whose expression levels in a cell are enhanced in response to a stressful stimulus. The purification of stress proteins  
35 belonging to these three families is described below.

The immunogenic hsp-peptide complexes of the invention include any complex containing an hsp and a peptide that is

capable of inducing immunotolerance in a mammal. The peptides are preferably noncovalently associated with the hsp. Preferred complexes include, but are not limited to, hsp90-peptide and hsp70-peptide complexes. For example, an hsp called gp96 which is present in the endoplasmic reticulum of eukaryotic cells and is related to the cytoplasmic hsp90's (i.e., is a member of the hsp90 family) can be used to generate an effective vaccine containing a gp96-peptide complex. In a specific embodiment, hsps complexed to the peptides with which they are endogenously associated are used, rather than hsps not so complexed, for purposes of convenience since the endogenous peptides copurify with the hsps. In another specific embodiment, the hsp and complexed antigen are both natively (i.e., non-recombinantly) produced in the same cell type.

In a specific embodiment the hsp used in accordance with the invention is not an alloantigen of the grafted tissue against which a graft rejection response may be elicited. In addition, in a specific embodiment for hsp-peptide complexes used in accordance with the invention, the complexed peptide is not an alloantigen of the grafted tissue against which a graft rejection response may be elicited. For example, and not by way of limitation, an autologous hsp-peptide complex would be substantially free of any alloantigen. In another specific embodiment, the autologous hsp-peptide complex is isolated from a healthy organ, such as liver, from a subject not experiencing either graft rejection or autoimmune response directed at that organ.

Although the hsps can be allogeneic to the patient, in a specific embodiment, the hsps are autologous to (derived from) the patient to whom they are administered. In specific embodiments, either the hsp, the complexed peptide, or both, are not obtained from the graft or transplant donor. In further specific embodiments, either the hsp, the complexed peptide, or both, are not syngeneic to the donor of the graft or transplant.

In additional specific embodiments, the hsp or hsp-peptide complex is not concomitantly used (e.g., not

administered with) additional molecules, such as antibodies, including monoclonal antibodies, or soluble receptors or soluble receptor analogues, that may contact and/or effectively modify the functional capabilities of immune system cells, such as antigen presenting cells, with which the hsp may come into contact.

The hsps and/or antigenic molecules can be purified from natural sources, chemically synthesized, or recombinantly produced. The invention provides methods for determining doses for treatment and prevention of graft rejection by evaluating the optimal dose of hsp, both unbound and noncovalently bound to peptide, in experimental animal models and extrapolating the data.

In a specific embodiment of the invention, the graft or transplant is allogeneic to the individual recipient. In another specific embodiment, the graft or transplant is xenogeneic to the recipient. For example, and not by way of limitation, human recipients may receive grafts or transplants from non-human mammalian donors, including but not limited to pigs and sheep.

The methods disclosed herein encompass the prevention and treatment of graft rejection in human and non-human transplant recipients, including but not limited to non-human mammals such as dogs, cats, and horses.

The therapeutic regimens and pharmaceutical compositions of the invention can be used with additional immune response enhancers or biological response modifiers including, but not limited to, the cytokines IFN- $\alpha$ , IFN- $\gamma$ , IL-2, IL-4, IL-6, TNF, or other cytokine affecting immune cells. In accordance with this aspect of the invention, the hsp either uncomplexed or complexed with antigenic molecule is administered in combination therapy with one or more of these cytokines.

The therapeutic regimens and pharmaceutical compositions of the invention can be used with additional immunosuppressants or biological response modifiers including, but not limited to, cyclosporine, azathioprine, mycophenolate mofetil, tacrolimus, corticosteroids, prednisone, cyclophosphamide, and antilymphocytes such as antilymphocyte globulin (ALG), antithymocyte globulin (ATG),

and orthoclone OKT3. The characteristics and use of such immunosuppressants are described in detail in First, 1998, "Clinical Application of Immunosuppressive Agents in Renal Transplantation," in The Surgical Clinics of America,

5 Venkateswara, K.R., ed., Vol. 78, No. 1 (W.B. Saunders Company: Philadelphia) at pages 61-76; and Chan, G.L.C., et al., 1990, "Principles of Immunosuppression," in Critical Care Clinics, October 1990, Vol. 6, No. 4 (W.B. Saunders Company: Philadelphia) at pages 841-892, each of which is  
10 hereby incorporated by reference in its entirety. In accordance with this aspect of the invention, the hsp either uncomplexed or complexed with antigenic molecule is administered in combination therapy with one or more of these immunosuppressants.

15 Accordingly, the invention provides methods of preventing and treating graft rejection in an individual comprising administering a composition which elicits specific immunotolerance to the target host cells or tissue.

#### 5.1 Grafted Cells, Tissues and Organs

20 Grafted cells, tissues, and organs whose rejection by recipient can be treated and prevented by the methods of the present invention include, but are not limited to, skin, liver, kidney, heart, bone marrow, pancreas, lung, cornea, and cartilage, and cells obtained from these tissues and  
25 organs, including but not limited to pancreatic islet cells.

#### 5.2 Obtaining Therapeutic Compositions for Treatment and Prevention of Graft Rejection

The hsps used in accordance with the invention can be complexed with antigenic molecules (e.g., peptides), or  
30 uncomplexed. Whether complexed or not, the hsps can be native (non-recombinant) or recombinant. The antigenic molecules can be endogenous, i.e., naturally associated with hsp intracellularly. Alternatively, the antigenic molecules can be exogenous, i.e., not naturally occurring in a  
35 noncovalent complex with hsps, or eluted from a cellularly derived noncovalent complex with hsps and reconstituted with

other hsps in vitro. Preferably, the hsp, or complex, as the case may be, is used in purified form, preferably to homogeneity as viewed on a polyacrylamide gel, or to at least 60%, 70%, 80%, or 90% of total protein.

- 5       The hsp-peptide complexes can be isolated as such from cells wherein the hsp and antigenic molecule are produced. Hsps or exogenous antigenic molecules can be produced in the cell by recombinant expression of a gene encoding that component (either hsp or antigenic molecule), or can be
- 10       isolated from native sources. The hsps and exogenous antigenic molecule components can be produced and isolated independently and complexed *in vitro*. Alternatively, complexes of hsps and endogenous peptides can be isolated from cells. In a preferred embodiment for *in vitro*
- 15       complexing of hsps and exogenous antigenic molecules, the hsp component is first isolated from cells as a complex, and then purified away from the noncovalently bound endogenous peptide with which it is complexed, prior to complexing *in vitro* with the exogenous antigenic molecule of interest. Alternatively,
- 20       the hsp component is first isolated from cells as a complex, and then the noncovalently bound endogenous peptide with which it is complexed is exchanged *in vitro* with the exogenous antigenic molecule of interest.

- 25       Accordingly, the protocols described herein can be used to isolate and produce purified hsps or purified complexes of hsps and antigenic molecules.

- 30       Uncomplexed endogenous hsps and endogenous hsps complexed with antigenic molecules can be isolated from any eukaryotic cells, including but not limited to, tissues, isolated cells, and immortalized eukaryotic cell lines. The tissue source need not be the same as the tissue which is targeted by the subject graft. Suitable source tissues include, but are not limited to liver, pancreas, or any other organ of mammalian or non-mammalian origin.

- 35       Alternatively, the hsps can be produced by recombinant DNA technology using techniques well known in the art. These methods are described in detail in Section 5.2.2, below.

Peptides derived from either a naturally expressed protein (i.e., native peptide) or from a recombinantly expressed protein can be isolated by first isolating the corresponding hsp-peptide complex and then eluting the peptide. Methods for eluting noncovalently bound peptide from the hsp-peptide complex are described in Section 5.2.4, below. Peptides can also be produced synthetically and subsequently complexed with hsps *in vitro*.

Methods for complexing hsps with antigenic molecules *in vitro* are described in Section 5.2.5, below.

The hsps to be used therapeutically, alone or complexed, can but need not be isolated from a sample from the patient to which they are then to be administered to treat or prevent graft rejection, i.e., the hsps (and antigenic molecules) can be autologous or non-autologous.

#### 5.2.1. Preparation of Hsp-Peptide Complexes

The methods described in Sections 5.2.1.1-5.2.1.3, below, can be used to isolate hsps complexed with antigenic molecules from cells, preferably from cells expressing non-recombinant hsps, although cells expressing recombinant hsps may also be used. A population of purified hsp-peptide complexes, comprising different peptides, can thus be obtained. These same methods may also be used to prepare purified hsp, by removing the endogenous antigenic molecules from the isolated complexes by methods described in Section 5.2.3, below.

##### 5.2.1.1. Preparation and Purification of gp96-peptide Complexes

Complexes of gp96 noncovalently bound to peptide can be readily obtained according to the procedure set forth in Srivastava et al., 1986, Proc. Natl. Acad. Sci. USA 83: 3407-3411, which is hereby incorporated by reference in its entirety.

A procedure that can be used, presented by way of example and not limitation, is as follows:

A pellet of eukaryotic cells (e.g., from liver, pancreas, or any other convenient organ) is resuspended in 3 volumes of buffer consisting of 30mM sodium bicarbonate buffer (pH 7.5) and 1mM PMSF and the cells allowed to swell on ice 20 minutes. The cell pellet then is homogenized in a Dounce homogenizer (the appropriate clearance of the homogenizer will vary according to each cells type) on ice until >95% cells are lysed.

The lysate is centrifuged at 1,000Xg for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step then is recentrifuged at 100,000Xg for 90 minutes. The gp96-peptide complex can be purified either from the 100,000Xg pellet or from the supernatant.

When purified from the supernatant, the supernatant is diluted with equal volume of 2X lysis buffer and the supernatant mixed for 2-3 hours at 4°C with Con A-Sepharose® (Pharmacia, Inc., Sweden) equilibrated with PBS containing 2mM  $\text{Ca}^{2+}$  and 2mM  $\text{Mg}^{2+}$ . Then, the slurry is packed into a column and washed with 1X lysis buffer until the  $\text{OD}_{280}$  drops to baseline. Then, the column is washed with 1/3 column bed volume of 10%  $\alpha$ -methyl mannoside ( $\alpha$ -MM) dissolved in PBS containing 2mM  $\text{Ca}^{2+}$  and 2mM  $\text{Mg}^{2+}$ , the column sealed with a piece of parafilm, and incubated at 37°C for 15 minutes. Then the column is cooled to room temperature and the parafilm removed from the bottom of the column. Five column volumes of the  $\alpha$ -MM buffer are applied to the column and the eluate analyzed by SDS-PAGE. Typically the resulting material is about 60-95% pure, however this depends upon the cell type and the tissue-to-lysis buffer ratio used. Then the sample is applied to a Mono Q® FPLC ion -exchange chromatographic column (Pharmacia, Inc., Piscataway, NJ) equilibrated with a buffer containing 5mM sodium phosphate, pH 7. The proteins then are eluted from the column with a 0-1M NaCl gradient and the gp96 fraction elutes between 400mM and 550mM NaCl.

The procedure, however, can be modified by two additional steps, used either alone or in combination, to

consistently produce apparently homogeneous gp96-peptide complexes. One optional step involves an ammonium sulfate precipitation prior to the Con A purification step and the other optional step involves DEAE-Sepharose® purification after the Con A purification step but before the Mono Q® FPLC step.

In the first optional step, the supernatant resulting from the 100,000Xg centrifugation step is brought to a final concentration of 50% ammonium sulfate by the addition of ammonium sulfate. The ammonium sulfate is added slowly while gently stirring the solution in a beaker placed in a tray of ice water. The solution is stirred from about  $\frac{1}{2}$  to 12 hours at 4°C and the resulting solution centrifuged at 6,000 rpm (Sorvall SS34 rotor). The supernatant resulting from this step is removed, brought to 70% ammonium sulfate saturation by the addition of ammonium sulfate solution, and centrifuged at 6,000 rpm (Sorvall SS34 rotor). The resulting pellet from this step is harvested and suspended in PBS containing 70% ammonium sulfate in order to rinse the pellet. This mixture is centrifuged at 6,000 rpm (Sorvall SS34 rotor) and the pellet dissolved in PBS containing 2mM  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Undissolved material is removed by a brief centrifugation at 15,000 rpm (Sorvall SS34 rotor). Then, the solution is mixed with Con A Sepharose® and the procedure followed as before.

In the second optional step, the gp96 containing fractions eluted from the Con A column are pooled and the buffer exchanged for 5mM sodium phosphate buffer, pH 7, 300mM NaCl by dialysis, or preferably by buffer exchange on a Sephadex® G25 column (Pharmacia, Inc., Sweden). After buffer exchange, the solution is mixed with DEAE-Sepharose® previously equilibrated with 5mM sodium phosphate buffer, pH 7, 300mM NaCl. The protein solution and the beads are mixed gently for 1 hour and poured into a column. Then, the column is washed with 5mM sodium phosphate buffer, pH 7, 300mM NaCl, until the absorbance at 280nm drops to baseline. Then, the bound protein is eluted from the column with five volumes of 5mM sodium phosphate buffer, pH 7, 700mM NaCl. Protein containing fractions are pooled and diluted with 5mM sodium phosphate buffer, pH 7 in order to lower the salt



concentration to 175mM. The resulting material then is applied to the Mono Q® FPLC column (Pharmacia) equilibrated with 5mM sodium phosphate buffer, pH 7 and the protein that binds to the Mono Q® FPLC column (Pharmacia) is eluted as described before.

It is appreciated, however, that one skilled in the art can assess, by routine experimentation, the benefit of incorporating the second optional step into the purification protocol. In addition, it is appreciated also that the benefit of adding each of the optional steps will depend upon the source of the starting material.

When the gp96 fraction is isolated from the 100,000Xg pellet, the pellet is suspended in 5 volumes of PBS containing either 1% sodium deoxycholate or 1% octyl glucopyranoside (but without the  $Mg^{2+}$  and  $Ca^{2+}$ ) and incubated on ice for 1 hour. The suspension is centrifuged at 20,000Xg for 30 minutes and the resulting supernatant dialyzed against several changes of PBS (also without the  $Mg^{2+}$  and  $Ca^{2+}$ ) to remove the detergent. The dialysate is centrifuged at 100,000Xg for 90 minutes, the supernatant harvested, and calcium and magnesium are added to the supernatant to give final concentrations of 2mM, respectively. Then the sample is purified by either the unmodified or the modified method for isolating gp96-peptide complex from the 100,000Xg supernatant, see above.

The gp96-peptide complexes can be purified to apparent homogeneity using this procedure. About 10-20 $\mu$ g of gp96-peptide complex can be isolated from 1g cells/tissue.

#### 5.2.1.2. Preparation and Purification of Hsp 70-peptide Complexes

The purification of hsp70-peptide complexes has been described previously, see, for example, Udono et al., 1993, *J. Exp. Med.* 178:1391-1396. A procedure that can be used, presented by way of example but not limitation, is as follows:

Initially, cells (e.g., from liver, pancreas, or any other convenient organ) are suspended in 3 volumes of 1X lysis buffer consisting of 5mM sodium phosphate buffer, pH 7, 150mM NaCl, 2mM  $\text{CaCl}_2$ , 2mM  $\text{MgCl}_2$  and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells can be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000Xg for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000Xg for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose® equilibrated with phosphate buffered saline (PBS) containing 2mM  $\text{Ca}^{2+}$  and 2mM  $\text{Mg}^{2+}$ . When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X lysis buffer prior to mixing with Con A Sepharose®. The supernatant is then allowed to bind to the Con A Sepharose® for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q® FPLC column equilibrated in 20mM Tris-Acetate pH 7.5, 20mM NaCl, 0.1mM EDTA and 15mM 2-mercaptoethanol. The column is then developed with a 20mM to 500mM NaCl gradient and then eluted fractions fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by immunoblotting using an appropriate anti-hsp70 antibody (such as from clone N27F3-4, from StressGen, Victoria, British Columbia, Canada).

Fractions strongly immunoreactive with the anti-hsp70 antibody are pooled and the hsp70-peptide complexes precipitated with ammonium sulfate; specifically with a 50%-

70% ammonium sulfate cut. The resulting precipitate is then harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. The washed precipitate is then solubilized and any residual ammonium sulfate removed by gel filtration on a Sephadex® G25 column (Pharmacia). If necessary the hsp70 preparation thus obtained can be repurified through the Mono Q® FPLC column as described above.

The hsp70-peptide complex can be purified to apparent homogeneity using this method. Typically 1mg of hsp70-peptide complex can be purified from 1g of cells/tissue.

The present invention further describes a rapid method for purification of hsp70-peptide complexes. This improved method comprises contacting cellular proteins with ADP or a nonhydrolyzable analog of ATP affixed to a solid substrate, such that hsp70 in the lysate can bind to the ADP or nonhydrolyzable ATP analog, and eluting the bound hsp70. A preferred method uses column chromatography with ADP affixed to a solid substratum (e.g., ADP-agarose). The resulting hsp70 preparations are higher in purity and devoid of contaminating peptides. The hsp70 yields are also increased significantly by about more than 10 fold. Alternatively, chromatography with nonhydrolyzable analogs of ATP, instead of ADP, can be used for purification of hsp70-peptide complexes.

By way of example but not limitation, purification of hsp70-peptide complexes by ADP-agarose chromatography is carried out as follows:

500 million cells (e.g., from liver, pancreas, or any other convenient organ) are homogenized in hypotonic buffer and the lysate is centrifuged at 100,000Xg for 90 minutes at 4°C. The supernatant is applied to an ADP-agarose column. The column is washed in buffer and is eluted with 5 column volumes of 3 mM ADP. The hsp70-peptide complexes elute in fractions 2 through 10 of the total 15 fractions which elute. The eluted fractions are analyzed by SDS-PAGE. The hsp70-peptide complexes can be purified to apparent homogeneity using this procedure.

### 5.2.1.3. Preparation and Purification of Hsp 90-peptide Complexes

A procedure that can be used to prepare hsp90-peptide complexes, presented by way of example and not limitation, is as follows:

Initially, cells (e.g., from liver, pancreas, or any other convenient organ) are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM CaCl<sub>2</sub>, 2mM MgCl<sub>2</sub> and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells can be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000Xg for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000Xg for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose® equilibrated with PBS containing 2mM Ca<sup>2+</sup> and 2mM Mg<sup>2+</sup>. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose®. The supernatant is then allowed to bind to the Con A Sepharose® for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 20 mM sodium phosphate, pH 7.4, 1mM EDTA, 250mM NaCl, 1mM PMSF. Then the dialysate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q® FPLC column equilibrated with lysis buffer. The proteins are then eluted with a salt gradient of 200mM to 600mM NaCl.

The eluted fractions are fractionated by SDS-PAGE and fractions containing the hsp90-peptide complexes identified by immunoblotting using an anti-hsp90 antibody such as 3G3

(Affinity Bioreagents). Hsp90-peptide complexes can be purified to apparent homogeneity using this procedure. Typically, 150-200  $\mu$ g of hsp90-peptide complex can be purified from 1g of cells/tissue.

#### 5 5.2.2. Recombinant Production of Hsps

Many genes encoding hsps have been cloned and sequenced, including, for example, human hsp70 (GenBank Accession Nos. M11717 and M15432; see also Hunt and Morimoto, 1985, Proc. Natl. Acad. Sci. USA 82: 6455-6459), human hsp90 (GenBank  
10 Accession No. X15183; see also Yamazaki et al., 1989, Nucleic Acids Res. 17: 7108), and human gp96 (GenBank Accession No. M33716; see also Maki et al., 1990, Proc. Natl. Acad. Sci. USA 87: 5658-5662).

The hsps can be produced by recombinant DNA technology  
15 using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing hsp coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, in vitro  
20 recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, *supra*, and Ausubel et al., 1989, *supra*.

A variety of host-expression vector systems can be  
25 utilized to express the hsp genes. These include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the hsp coding sequence; yeast (e.g. *Saccharomyces*, *Pichia*)  
30 transformed with recombinant yeast expression vectors containing the hsp coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the hsp coding sequence; plant cell systems infected with recombinant virus expression vectors  
35 (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the hsp coding

sequence; or mammalian cell systems (e.g. COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the hsp coding sequence can be ligated individually into the vector in frame with the *lacZ* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned hsp gene protein can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The hsp gene can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the hsp coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems can be utilized. In cases where an adenovirus is used as an expression vector, the hsp coding sequence can be ligated to an adenovirus

5 transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a  
10 recombinant virus that is viable and capable of expressing hsps in infected hosts. (See, e.g., Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted hsp coding sequence. These signals  
15 include the ATG initiation codon and adjacent sequences. The efficiency of expression can be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

20 In addition, a host cell strain can be chosen which modulates the expression of the inserted sequences, or modifies and processes the hsp in the specific fashion desired. For example, choosing a system that allows for appropriate glycosylation is especially important in the case  
25 of gp96. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins such as glycosylation. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein  
30 expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3,  
35 WI38, etc.

In a preferred embodiment for recombinant expression of hsps, the histidine-nickel (his-Ni) tag system is used (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:

8972-8976). In the his-Ni system, the hsp is expressed in human cell lines as a fusion protein which can be readily purified in a non-denatured form. In this system, the gene of interest (i.e., the hsp gene) is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto  $\text{Ni}^{2+}$ -nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Kits for expressing and isolating proteins using the his-Ni system are commercially available from Invitrogen®, San Diego, California.

Alternatively, recombinant hsps produced in eukaryotic hosts cells as described in this section, above, can be purified according to the respective methods detailed in Section 5.2.1, above.

#### 5.2.3. Preparation and Purification of Uncomplexed hsps

The following methods can be used to obtain uncomplexed hsps, i.e., hsps that are substantially free of noncovalently bound antigenic molecules such as peptides. The hsps can be administered in their uncomplexed form in accordance with the invention for the treatment and prevention of graft rejection. In addition, the uncomplexed hsps can be used to design hsp-antigenic molecule complexes by complexing them *in vitro* with antigenic molecules of interest, as described in Section 5.2.5, below.

##### 5.2.3.1. General Methods

Methods which can be used to separate the hsp and antigenic molecule components of the hsp-antigenic molecule complexes from each other, include, but are not limited to, treatment of the complexes with low pH. The low pH treatment methods described in this section, below, can be used for hsp70, hsp90, or gp96. An alternative method which is



preferred for isolating hsp70 from hsp-antigenic molecule complexes is provided in Section 5.2.3.2.

By way of example but not limitation, to elute the noncovalently bound antigenic molecule using low pH, acetic acid or trifluoroacetic acid is added to the purified hsp-antigenic molecule complex to give a final concentration of 10% (vol/vol) and the mixture incubated at room temperature or in a boiling water bath or any temperature in between, for 10 minutes (See, Van Bleek, et al., 1990, *Nature* 348:213-216; and Li, et al., 1993, *EMBO Journal* 12:3143-3151). The resulting samples are centrifuged through a Centricon® 10 assembly. The high and low molecular weight fractions are recovered. The remaining large molecular weight hsp70--peptide complexes can be reincubated in low pH to remove any remaining peptides. The resulting higher molecular weight fractions containing hsp are pooled and concentrated.

#### 5.2.3.2 Preferred Method for Preparation and Purification of Un-complexed Hsp 70

Preferably, the hsp70-peptide complex is purified as described above in Section 5.2.1.2. Once the hsp70-peptide complex is purified, the peptide is eluted from the hsp70 by either of the following two preferred methods. More preferably, the hsp70-peptide complex is incubated in the presence of ATP. Alternatively, the hsp70-peptide complex is incubated in a low pH buffer, as described in Section 5.2.2, above.

Briefly, the complex is centrifuged through a Centricon® 10 assembly (Millipore) to remove any low molecular weight material loosely associated with the complex. The large molecular weight fraction can be removed and analyzed by SDS-PAGE while the low molecular weight can be analyzed by HPLC as described below. In the ATP incubation protocol, the stress protein-peptide complex in the large molecular weight fraction is incubated with 10mM ATP for 30 minutes at room temperature.

The resulting samples are centrifuged through a Centricon® 10 assembly as mentioned previously. The high and

low molecular weight fractions are recovered. The remaining large molecular weight hsp70-peptide complexes can be reincubated with ATP to remove any remaining peptides.

The resulting higher molecular weight fractions  
5 containing hsp70 are pooled and concentrated.

#### 5.2.4 Isolation of Antigenic Components

The methods described in Section 5.2.3, above, which can be used to isolate hsps from complexes with antigenic molecules, can similarly be used to isolate peptides and/or  
10 antigenic components from cells which may contain potentially useful antigenic determinants. Once the hsps and antigenic molecules are separated from each other into separate fractions, the fractions containing the antigenic molecules can be pooled and processed further, as described below.  
15 Once isolated, the amino acid sequence of each antigenic peptide can be determined using conventional amino acid sequencing methodologies. Such antigenic molecules can then be produced by chemical synthesis or recombinant methods, purified, and complexed to hsps *in vitro*.

20 Similarly, it has been found that potentially immunogenic peptides may be eluted from MHC-peptide complexes using techniques well known in the art (Falk, K. et al., 1990 *Nature* 348:248-251; Elliott, T., et al., 1990, *Nature* 348:195-197; Falk, K., et al., 1991, *Nature* 351:290-296).

25 Thus, potentially immunogenic or antigenic peptides can be isolated from either stress protein-peptide complexes or MHC-peptide complexes for use subsequently as antigenic molecules, by complexing *in vitro* to hsps. Exemplary protocols for isolating peptides and/or antigenic components  
30 from either of the these complexes are set forth below in Sections 5.2.4.1 and 5.2.4.2.

##### 5.2.4.1. Peptides From Stress Protein-Peptide Complexes

The methods detailed in Section 5.2.3, above, can be  
35 used to elute the peptide from a stress protein-peptide complex. One approach involves incubating the stress

protein-peptide complex in the presence of ATP. The other approach involves incubating the complexes in a low pH buffer.

5 Briefly the complex of interest is centrifuged through a Centricon® 10 assembly (Millipore) to remove any low molecular weight material loosely associated with the complex. The large molecular weight fraction can be removed and analyzed by SDS-PAGE while the low molecular weight can be analyzed by HPLC as described below. In the ATP  
10 incubation protocol, the stress protein-peptide complex in the large molecular weight fraction is incubated with 10mM ATP for 30 minutes at room temperature. In the low pH protocol, acetic acid or trifluoroacetic acid (TFA) is added to the stress protein-peptide complex to give a final  
15 concentration of 10% (vol/vol) and the mixture incubated at room temperature or in a boiling water bath or any temperature in between, for 10 minutes (See, Van Bleek, et al., 1990, *Nature* 348:213-216; and Li, et al., 1993, *EMBO Journal* 12:3143-3151).

20 The resulting samples are centrifuged through a Centricon® 10 assembly as mentioned previously. The high and low molecular weight fractions are recovered. The remaining large molecular weight stress protein-peptide complexes can be reincubated with ATP or low pH to remove any remaining  
25 peptides.

The resulting lower molecular weight fractions are pooled, concentrated by evaporation and dissolved in 0.1% TFA. The dissolved material is then fractionated by reverse phase high pressure liquid chromatography (HPLC) using for  
30 example a VYDAC® C18 reverse phase column (Separations Group, Inc., Hesperia, CA) equilibrated with 0.1% TFA. The bound material is then eluted at a flow rate of about 0.8 ml/min by developing the column with a linear gradient of 0 to 80% acetonitrile in 0.1% TFA. The elution of the peptides can be  
35 monitored by OD<sub>210</sub> and the fractions containing the peptides collected.

#### 5.2.4.2. Peptides from MHC-peptide Complexes

The isolation of potentially immunogenic peptides from MHC molecules is well known in the art and so is not described in detail herein (See, Falk, et al., 1990, *Nature* 348:248-251; Rotzsche, et al., 1990, *Nature* 348:252-254; Elliott, et al., 1990, *Nature* 348:191-197; Falk, et al., 1991, *Nature* 351:290-296; Demotz, et al., 1989, *Nature* 343:682-684; Rotzsche, et al., 1990, *Science* 249:283-287, the disclosures of which are incorporated herein by reference).

Briefly, MHC-peptide complexes can be isolated by a conventional immunoaffinity procedure. The peptides then can be eluted from the MHC-peptide complex by incubating the complexes in the presence of about 0.1% TFA in acetonitrile. The eluted peptides can be fractionated and purified by reverse phase HPLC, as before.

The amino acid sequences of the eluted peptides can be determined either by manual or automated amino acid sequencing techniques well known in the art. Once the amino acid sequence of a potentially protective peptide has been determined the peptide can be synthesized in any desired amount using conventional peptide synthesis or other protocols well known in the art.

#### 5.2.4.3. Synthetic Production of Peptides

Peptides having the same amino acid sequence as those isolated above can be synthesized by solid-phase peptide synthesis using procedures similar to those described by Merrifield, 1963, *J. Am. Chem. Soc.*, 85:2149. During synthesis, N- $\alpha$ -protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to an insoluble polymeric support i.e., polystyrene beads. The peptides are synthesized by linking an amino group of an N- $\alpha$ -deprotected amino acid to an  $\alpha$ -carboxy group of an N- $\alpha$ -protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free

amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N- $\alpha$ -protecting groups include Boc which is acid labile and Fmoc which is base labile.

5 Briefly, the C-terminal N- $\alpha$ -protected amino acid is first attached to the polystyrene beads. The N- $\alpha$ -protecting group is then removed. The deprotected  $\alpha$ -amino group is coupled to the activated  $\alpha$ -carboxylate group of the next N- $\alpha$ -protected amino acid. The process is repeated until the  
10 desired peptide is synthesized. The resulting peptides are then cleaved from the insoluble polymer support and the amino acid side chains deprotected. Longer peptides can be derived by condensation of protected peptide fragments. Details of appropriate chemistries, resins, protecting groups, protected  
15 amino acids and reagents are well known in the art and so are not discussed in detail herein (See, Atherton, et al., 1989, *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press, and Bodanszky, 1993, *Peptide Chemistry, A Practical Textbook*, 2nd Ed., Springer-Verlag).

20 Purification of the resulting peptides is accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

25 5.2.5. *In Vitro* Production of Stress  
Protein-Antigenic Molecule Complexes

In an embodiment in which complexes of hsps and the peptides with which they are endogenously associated *in vivo* are not employed, and it is desired to use hsp-antigenic  
30 molecule complexes, complexes of hsps to antigenic molecules are produced *in vitro*. As will be appreciated by those skilled in the art, the peptides either isolated by the aforementioned procedures or chemically synthesized or recombinantly produced can be reconstituted with a variety of  
35 purified natural or recombinant stress proteins *in vitro* to generate immunogenic noncovalent stress protein-antigenic

molecule complexes. Alternatively, exogenous antigens or antigenic/immunogenic fragments or derivatives thereof can be noncovalently complexed to stress proteins for use in the immunotherapeutic or prophylactic vaccines of the invention.

- 5 A preferred, exemplary protocol for noncovalently complexing a stress protein and an antigenic molecule *in vitro* is discussed below.

Prior to complexing, the hsps are pretreated with ATP or low pH to remove any peptides that may be associated with the  
10 hsp of interest. When the ATP procedure is used, excess ATP is removed from the preparation by the addition of apyranase as described by Levy, et al., 1991, *Cell* 67:265-274. When the low pH procedure is used, the buffer is readjusted to neutral pH by the addition of pH modifying reagents.

- 15 The antigenic molecules (1 $\mu$ g) and the pretreated hsp (9 $\mu$ g) are admixed to give an approximately 5 antigenic molecule: 1 stress protein molar ratio. Then, the mixture is incubated for 15 minutes to 3 hours at 4° to 45°C in a suitable binding buffer such as one containing 20mM sodium  
20 phosphate, pH 7.2, 350mM NaCl, 3mM MgCl<sub>2</sub>, and 1mM phenyl methyl sulfonyl fluoride (PMSF). The preparations are centrifuged through a Centricon® 10 assembly (Millipore) to remove any unbound peptide. The association of the peptides with the stress proteins can be assayed by SDS-PAGE. This is the  
25 preferred method for *in vitro* complexing of peptides isolated from MHC-peptide complexes of peptides disassociated from endogenous hsp-peptide complexes.

- In an alternative embodiment of the invention, preferred for producing complexes of hsp70 to exogenous antigenic  
30 molecules such as peptides, 5-10 micrograms of purified hsp is incubated with equimolar quantities of the antigenic molecule in 20mM sodium phosphate buffer pH 7.5, 0.5M NaCl, 3mM MgCl<sub>2</sub> and 1mM ADP in a volume of 100 microliter at 37°C for 1 hr. This incubation mixture is further diluted to 1ml  
35 in phosphate-buffered saline.

In an alternative embodiment of the invention, preferred for producing complexes of hsp90 to peptides, 5-10 micrograms of purified hsp90 is incubated with equimolar or excess

quantities of the antigenic peptide in a suitable buffer such as one containing 20mM sodium phosphate buffer pH 7.5, 0.5M NaCl, 3mM MgCl<sub>2</sub> at 60-65°C for 5-20 min. Alternatively, equimolar or excess quantities of peptide (e.g., exogenous peptide) are added to purified hsp90-peptide (endogenous) complex, such that the exogenous peptide is exchanged for the endogenous peptide. In either case, the incubation mixture is allowed to cool to room temperature and centrifuged one or more times if necessary, through a Centricon® 10 assembly (Millipore) to remove any unbound peptide.

In an alternative embodiment of the invention, preferred for producing complexes of gp96 to peptides, 100-300nM purified peptide is added to 100nM purified gp96. Alternatively, 100-300nM peptide (e.g., exogenous peptide) is added to purified gp96-peptide (endogenous) complex, such that the exogenous peptide is exchanged for the endogenous peptide. In either case, the mixture is incubated in a binding buffer consisting of 20mM HEPES, pH 7.2, 20 mM NaCl, and 2mM MgCl<sub>2</sub> at 60°C for 10 min. and allowed to cool to room temperature for an additional 10 min. After centrifugation, the sample is incubated for 30 min. at room temperature. Free peptide is removed completely using a microcon 50 (Amicon, Inc.).

Once complexes have been isolated, they can be characterized further for tolerogenicity in animal models using the preferred administration protocols and excipients discussed below.

#### 5.2.6. Hsp Covalently Linked To Peptide

In addition to the non-covalent complexes of hsp and peptide described above, hsp covalently linked (i.e., covalently coupled or joined) to peptide may be used in accordance with the invention to inhibit graft rejection. For example, and not by way of limitation, hsp and peptide can be prepared separately according to the methods described in Sections 5.2.3-5.2.4, above. Free peptide can then be covalently linked to hsp by mixing each component in the presence of a cross-linking agent, including but not limited

to glutaraldehyde. Such covalently linked hsp-peptide complexes can be made using, for example, the method of Lussow et al., 1991, Eur. J. Immunol. 21:2297-2302 and Barrios et al., 1992, Eur. J. Immunol. 22:1365-1372, each of which is hereby incorporated by reference in its entirety.

Alternatively, a peptide can be covalently linked to an hsp by genetically engineering an hsp-peptide fusion protein, using recombinant DNA techniques well known in the art. More specifically, the coding sequence of an hsp can be obtained as described in Section 5.2.2, above, for example, and then fused to a DNA sequence coding for a peptide. This construct can then be expressed in a host cell and purified as an intact fusion protein, using the methods described in Section 5.2.2, above, for example. Preferably, the peptide is fused to the peptide binding domain of the hsp.

### 5.3 Dosage Regimens

Hsps and hsp-antigenic molecule complexes are administered to mammalian subjects, e.g., primates, dogs, cats, mice, rats, horses, cows, pigs, etc., preferably humans, in doses in a range of about 5  $\mu$ g to about 5000  $\mu$ g, alternatively in a range of about 5  $\mu$ g to about 1500  $\mu$ g. In an additional embodiment for mammals, a range of about 50  $\mu$ g to about 500  $\mu$ g, either intradermally or subcutaneously may be used. Alternatively a range of about 50  $\mu$ g to about 200  $\mu$ g subcutaneously and about 5  $\mu$ g to about 100  $\mu$ g intradermally may be used. Thus, while both subcutaneous and intradermal routes of administration are effective, intradermal injections typically require a lower dosage and are, therefore, preferred with respect to economy of materials.

As demonstrated in the examples in Sections 6 and 7, below, an effective dose for prevention of graft rejection in mice is 100  $\mu$ g and 200  $\mu$ g gp96 subcutaneously for mice of average mass of 20-25 g. These amounts of hsp (100-200  $\mu$ g range) are high compared to the relatively small amounts of hsp-peptide complex that are required to elicit an effective immune response against an antigenic peptide, such as a



complexed tumor antigen. Similar high dosages of 100-200  $\mu$ g, or more than 200  $\mu$ g, of hsp may also be effective in treatment of larger mammals, including humans.

5 Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The hsp or complexes may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g.,  
10 oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local; this may be achieved, for example and not by way of limitation, by topical application, by injection, by means of a catheter, by  
15 means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In a specific embodiment, the hsp compositions are  
20 administered, either intradermally or subcutaneously, with sites of administration varied sequentially. For example, and not by way of limitation, the doses recited above are given once weekly for a period of about 4 to 6 weeks, and the mode of administration is varied with each administration.  
25 Each site of administration may be varied sequentially. Thus, by way of example and not limitation, the first injection can be given, either intradermally or subcutaneously, on the left arm, the second on the right arm, the third on the left belly, the fourth on the right belly,  
30 the fifth on the left thigh, the sixth on the right thigh, etc. The same site can be repeated after a gap of one or more injections. Also, split injections can be given. Thus, for example, half the dose can be given in one site and the other half in another site on the same day.

35 After 4-6 weeks, further injections are preferably given at two-week intervals over a period of time of one month. Later injections can be given monthly. The pace of later injections can be modified, depending upon the patient's

clinical progress and responsiveness to the immunotherapy. Alternatively, the mode of administration is sequentially varied, e.g., weekly administrations are given in sequence intradermally or subcutaneously.

5           5.4. Formulation

          The uncomplexed hsp's or hsp's complexed with antigenic molecules, in accordance with the invention, can be formulated into pharmaceutical preparations for administration to mammals, preferably humans, for treatment or prevention of graft rejection. In addition, immunosuppressive agents, as described in Section 5, above, can be formulated separately from or in combination with the hsp's and hsp-antigenic molecule complexes described herein for use in accordance with the invention. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier can be prepared, packaged, and labeled for treatment and prevention of rejection of grafted tissues and organs, such as skin, liver, kidney, heart, bone marrow, pancreas, lung, cornea, and cartilage.

20           If the complex is water-soluble, then it can be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it can be formulated with a non-ionic surfactant such as Tween, or polyethylene glycol. Thus, the compounds and their physiologically acceptable solvates can be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, rectal administration.

30           For oral administration, the pharmaceutical preparation can be in liquid form, for example, solutions, syrups or suspensions, or can be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents

(e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets can be coated by methods well-known in the art.

Preparations for oral administration can be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions can take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds can be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The

optionally further comprises in a container a pharmaceutically acceptable solution (e.g., saline, dextrose solution, etc.), preferably sterile, to reconstitute the complex to form a solution for injection purposes.

5 In another embodiment, a kit of the invention further comprises a needle or syringe, preferably packaged in sterile form, for injecting the complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of hsp or hsp-antigenic molecule complexes by a clinician or  
10 by the patient.

#### 5.5. Treatment and Prevention of Graft Rejection

The hsp-based compositions and formulations described above in Sections 5.2 and 5.4 can be used to treat or prevent graft rejection of cells, tissues, and organs,  
15 including but not limited to skin, liver, kidney, heart, bone marrow, pancreas, lung, cornea, and cartilage and cells derived therefrom.

##### 5.5.1 Methods of Treatment and Prevention Based on Administration of Hsps

20 The Examples presented in Sections 6 and 7, below, detail the use in accordance with the methods of the invention of the hsp gp96 in immunoprophylaxis in experimental skin graft rejection in mice. The administration of hsp effectively inhibited graft rejection  
25 when administered prior to transplantation.

Suppression of a rejection response may also be enhanced by administration of the hsp after transplantation. Transplantation may trigger an incipient graft rejection response. Administration of hsp after transplantation may  
30 specifically suppress such an activated rejection response.

In a specific embodiment, the treatment regimens provided herein comprise administration of the hsps after the onset of the graft rejection response; i.e., after the  
35 specific immune response has already developed. Hsp administration results in regulation of the activity of the relevant, pathologically active effector cells. Thus, the

compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds can also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds can also be formulated as a depot preparation. Such long acting formulations can be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

The compositions can, if desired, be presented in a pack or dispenser device which can contain one or more unit dosage forms containing the active ingredient. The pack can for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device can be accompanied by instructions for administration.

The invention also provides kits for carrying out the therapeutic regimens of the invention. Such kits comprise in one or more containers therapeutically or prophylactically effective amounts of the hsp or hsp-antigenic molecule complexes in pharmaceutically acceptable form. The hsp or hsp-antigenic molecule complex in a vial of a kit of the invention can be in the form of a pharmaceutically acceptable solution, e.g., in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the complex can be lyophilized or desiccated; in this instance, the kit

treatment methods of the present invention exploit not only the general properties of hsps but also the specificity of the naturally arisen pathological immune response.

Therefore, the treatment methods of the invention are more  
5 specific than common cytokine approaches to induction of suppression which are excessively systemic. The hsps used in accordance with the invention exert a more local and targeted immunosuppressive effect at the site of autoimmune cellular activity.

10 Thus, the invention encompasses administration of hsp before, after, or both before and after grafting or transplantation.

Pre-treatment of the recipient with a sample of donor tissue and hsp may be used, in accordance with the invention, to  
15 exploit the ability of hsps to specifically suppress an activated immune response. Thus, in another specific embodiment, the recipient may be pre-treated, prior to transplantation, with a tissue sample obtained from the donor organ. Preferably, this tissue is a dispensable sample which  
20 would not jeopardize the health of the recipient if rejected. Examples of such pre-treatment tissue include, but are not limited to, small portions of the actual tissue or organ to be transplanted. However, the tissue used in the pre-treatment need not be the same as the tissue to be  
25 transplanted. Thus, the pre-treatment tissue can be different than the tissue or organ to be transplanted. The key aspect of the pre-treatment tissue is that it expresses alloantigens of the donor. These alloantigens elicit a T cell response against the tissue of the donor. This response  
30 could potentially damage the eventual transplanted donor tissue or organ. However, in a specific embodiment of the invention, hsp is administered to the recipient after exposure to the tissue sample, but prior to actual transplantation. In this manner, the hsp specifically  
35 suppresses this response against the sample donor tissue. The tissue or organ of interest is then transplanted. When the transplanted tissue or organ is subsequently introduced into the recipient, the rejection response has already been suppressed by the donor tissue/hsp pre-treatment.

Thus, administration of hsp prior to transplantation may further comprise, in accordance with the invention, treatment of the recipient with a sample of donor tissue prior to administration of hsp.

5           5.6. Donor Selection and Tissue Typing

Donor tissues and organs for engraftment and transplantation can be selected using standard screening and tissue typing methods well known in the art, so as to minimize the likelihood of rejection. These methods include, but are not limited to, matching HLA phenotypes of donor and recipient which is well known in the art and described in detail in Valente et al., 1998 "Immunobiology of Renal Transplantation", in The Surgical Clinics of America, Venkateswara, K.R., ed., Vol. 78, No. 1 (W.B. Saunders Company: Philadelphia) at pages 1-26, which is hereby incorporated by reference in its entirety.

In addition, donors and recipients may be screened for suitability of transplantation to determine the extent of contraindications using the criteria detailed in Kasiske, 1998, "The Evaluation of Prospective Renal Transplant Recipients and Living Donors," in The Surgical Clinics of America, Venkateswara, K.R., ed., Vol. 78, No. 1 (W.B. Saunders Company: Philadelphia) at pages 27-39, which is hereby incorporated by reference in its entirety.

25       6.     EXAMPLE: PREVENTION OF SKIN GRAFT REJECTION IN MICE - EXPERIMENT 1

The experiment detailed below, referred to herein as Experiment 1, demonstrates the effectiveness of the heat shock protein gp96 in inhibiting graft rejection.

30       6.1 Materials and Methods

gp96 was isolated as a non-covalent gp96-peptide complex from liver and from skin of BALB/cJ (H-2<sup>d</sup>) mice (i.e., syngeneic with graft donor BALB/cJ (H-2<sup>d</sup>) mice), according to the method described Srivastava et al., 1986, Proc. Natl.

Acad. Sci. USA 83: 3407-3411. Purified gp96 (complexed with peptide) was suspended in phosphate buffered saline (PBS). Donor skin cell and liver cell lysates were obtained as a 100,000Xg supernatant prepared as described in Srivastava et al., 1986, *supra*.

Five different groups each consisting of five recipient C57BL/6(H-2<sup>b</sup>) were treated with subcutaneous injections on the first day (Day 0), as follows:

- 1) PBS (buffer alone);
- 2) 100 µg gp96 isolated from donor liver;
- 3) 100 µg gp96 isolated from donor skin;
- 4) 100 µg gp96 isolated from donor liver and 100 µg gp96 isolated from donor skin;
- 5) a mixture of donor liver and skin cell lysates.

Seven days later (on Day 7) each dose was repeated. Three days later (on Day 10), each C57BL/6(H-2<sup>b</sup>) recipient received a full thickness skin graft as follows.

Recipient C57BL/6(H-2<sup>b</sup>) mice were wounded by creating a wound of 1.2 cm in diameter, which expanded to 1.6 cm in diameter. An appropriately sized disk was marked with a pen, and full-thickness skin was excised using a scalpel.

Skin grafts were obtained from BALB/cJ (H-2<sup>d</sup>) donor mice by excising a 1.6 cm diameter patch of skin. Grafts were sewn onto the wound of recipient mice using 4.0 silk interrupted sutures. Grafts were meshed by making random incisions on the surface to allow seepage and prevent tenting of the graft.

Grafts were analyzed 4, 5, 7, 8, 9, and 10 days after engraftment (on Day 14, Day 15, Day 17, Day 18, Day 19, and Day 20, respectively).

## 6.2 Results

The results for all of Days 14, 15, 17, 18, and 19 are depicted in FIG. 1. Graft rejection was most effectively inhibited in the mice of group 2, which received the 100 µg gp96 isolated from liver. Graft rejection was also effectively inhibited in the mice of group 3, which received the 100 µg gp96 isolated from skin.



7. EXAMPLE: PREVENTION OF SKIN GRAFT REJECTION IN  
MICE - EXPERIMENT 2

The experiment detailed below, referred to herein as  
5 Experiment 2, also demonstrates the effectiveness of the heat  
shock protein gp96 in inhibiting graft rejection.

7.1. Materials and Methods

gp96 was isolated as a non-covalent gp96-peptide complex  
from liver of BALB/cJ (H-2<sup>d</sup>) mice, (i.e., syngeneic with graft  
10 donor BALB/cJ (H-2<sup>d</sup>) mice) or liver of Lewis rats, according to  
the method described in Srivastava et al., 1986, *supra*.

Purified gp96 (complexed with peptide) was suspended in PBS.

Ten different groups each consisting of two recipient  
C57BL/6(H-2<sup>b</sup>) were either untreated, treated with PBS  
15 (buffer), or treated with gp96 as follows:

- 1) No treatment;
- 2) Buffer administered intradermally;
- 3) Buffer administered subcutaneously;
- 4) 1  $\mu$ g gp96 administered intradermally;
- 20 5) 10  $\mu$ g gp96 administered intradermally;
- 6) 10  $\mu$ g gp96 administered subcutaneously;
- 7) 100  $\mu$ g gp96 administered intradermally;
- 8) 100  $\mu$ g gp96 administered subcutaneously;
- 9) 200  $\mu$ g gp96 administered subcutaneously;
- 25 10) 10  $\mu$ g rat gp96 administered intradermally.

Administrations were given at the "12 o'clock" and "6  
o'clock" positions of the roughly circular wounds.

Seven days later (Day 7), each dosage of gp96 or buffer  
was repeated. Skin grafts were carried out three days later  
30 (Day 10) as follows.

Recipient C57BL/6(H-2<sup>b</sup>) mice were wounded as described in  
Section 6.1, above.

Skin grafts were obtained from BALB/cJ (H-2<sup>d</sup>) donor mice  
as described in Section 6.1, above.

Grafts were analyzed 8, 12, 14, 16, and 19 days after engraftment (Day 18, Day 22, Day 24, Day 26, and Day 29, respectively).

#### 7.2. Results

5       The results for all of Days 18, 22, 24, 26, and 29 are summarized in FIG. 2. The results for Day 18 are depicted in FIGS. 3A-B. The results for Day 22 are depicted in FIGS. 4A-B. The results for Day 24 are depicted in FIGS. 5A-B. The results for Day 26 are depicted in FIGS. 6A-B. The results  
10       for Day 29 are depicted in FIGS. 7A-B.

      Rejection of the graft was clearly delayed in groups 8 and 9 (100  $\mu$ g and 200  $\mu$ g gp96, respectively, each administered subcutaneously). Rejection was most effectively delayed in group 9 (200  $\mu$ g gp96 administered subcutaneously).  
15       For example, mouse 1 in group 8 and mouse 1 in group 9 each had a healthy graft on Day 18 and Day 22. In addition, mouse 2 in group 8 and mouse 2 in group 9 each had a grafts that were much healthier than the grafts of all the mice from the other groups on Day 22.

20       The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such  
25       modifications are intended to fall within the scope of the appended claims.

      Various references including patent applications, patents, and other publications, are cited herein, the disclosures of which are incorporated by reference in their  
30       entireties.